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Inoculation Studies Related to Breeding for Resistance to Bacterial Wilt in Lespedeza

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Inoculation Studies Related to Breeding for Resistance to Bacterial Wilt in Lespedeza

M. S. Offutt and J. D. Baldridge*

INTRODUCTION

Bacterial wilt, caused by *Xanthomonas lespedezae* (Ayers *et al.*) Starr, is one of the most destructive diseases of the annual lespedezas in Missouri. It has been more damaging in Standard Korean lespedeza *(Lespedeza stipulacea*

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Fig. 1—Two common diseases of Korean lespedeza. The two plants on the left are healthy. The three plants in the middle are dying from bacterial wilt; note the wilting terminal leaves and the healthy roots. The three plants on the right are dying from root rot; note the wilting terminal leaves and stems and the decaying roots.

Maxium) than in common lespedeza (*Lespedeza striata* Thunb.).

The disease was first observed by Lefebvre *et al.* (5) in 1937 at the Arlington Experiment Farm, Arlington, Va. Subsequently, it was reported in Missouri, Iowa, Kansas, Illinois, Tennessee, and New York (1). Bacterial wilt now appears to be widespread in Missouri.

The first evidence of the seriousness of the disease in Missouri came from experimental plantings at the University of Missouri South Farms near Columbia. Bacterial wilt was prevalent in plantings of Korean lespedeza in nine years out of twelve during the period 1942 to 1953. In experimental pastures, Korean lespedeza has been increasingly difficult to maintain and the difficulty has been attributed in part to damage from wilt. The disease appears to be very destructive in old, volunteer stands subjected to heavy grazing. In strain tests of annual lespedeza, most of the strains, including Standard Korean, have been damaged frequently by wilt (Figure 1).

As the destructiveness of wilt became evident, the need for resistant strains of lespedeza was recognized. Breeding resistant strains offered the most logical means of controlling the disease, but suitable methods and procedures were not available.

It was the purpose of this investigation: (1) To develop inoculation procedures for determining the resistance to wilt of annual lespedeza strains and for selecting new resistant strains, (2) to study the effects of the disease on lespedeza strains, and (3) to determine some of the factors that influence the spread and severity of the disease.

REVIEW OF LITERATURE

Bacterial wilt of lespedeza was first identified and named by Lefebvre, Ayers, and Johnson (5) in 1939. Ayers, Lefebvre, and Johnson (1) found the disease to be a typical bacterial wilt of the vascular system.

The morphological and cultural characters of the causal organism *Xanthomonas lespedezae* (Ayers *et al.*) Starr have been studied in detail by Ayers *et al.* (1), Burkholder and Starr (3), and Starr (6). Ayers *et al.* (1) in 1939 described the pathogen as follows:

> The causal bacterium is a rod-shaped, Gram-negative, monotrichous organism that digests starch vigorously and grows especially well on starchy media. Colonies on agar are primuline yellow and those on nutrient agar are appressed and extremely viscid. The organism liquefies gelatin, egg albumin, and blood serum and forms both hydrogen sulfide and indol. Nitrates are not reduced and neither acid nor gas are (is) formed from dextrose, lactose, maltose, saccharose, starch, and mannitol. Milk becomes alkaline and cleared. Optimum temperature for growth of the organism in culture is near 35° C.

The lespedeza wilt pathogen was considered by Ayers *et al.* (1) to be an undescribed species of bacterium,and the name *Phytomonas lespedezae,*

n. sp. was proposed by them in 1939. In 1946, the lespedeza parasite was reclassified and placed in the genus *Xanthomonas* by Starr (6).

The mode of entry of the parasite into the host was partially investigated by Ayers *et al.* (1). They suggested that the bacterium could make its way through parenchyma from wounds or from stomata to the vascular tissue.

According to Ayers *et al.* (1), once the wilt organism has gained entrance into the vascular system it can travel rapidly in the xylem vessels and, apparently, can pass without difficulty from the leaves to the base of the stem or from the roots to the stem.

Overwintering of the pathogen has not been studied extensively, but Ayers *et at.* (1) point out that since farmers usually permit the annual lespedezas to reseed year after year in the same fields, infected seed and the trash from diseased plants may harbor the bacterium during the winter months. As evidence of this, they cited experiments made at the Arlington Farm. They were able to isolate the parasite from seed harvested from diseased plants and from old, diseased plant stems collected from the field as late as April 14.

Brown and Baldridge (10, 11, 12) stated that wet weather during the spring and early summer, when accompanied by heavy grazing, might be a factor contributing to the spread and to the severity of the disease in the field.

For practical control of the disease, Ayers *et al.* (1) have suggested breeding of resistant varieties. Wilsie and Hughes (7), Helm and McHoney (8), and Hanson (9) made limited attempts to locate wilt resistant strains of annual lespedeza. The results were not wholly satisfactory because of the lack of suitable procedures for selecting strains with a high degree of resistance.

Feaster (4) has pointed out that one of the main obstacles in investigating disease resistance has been that of successfully obtaining the same degree of infection on given genotypes each time it is attempted. Breeding for resistance is facilitated by inoculation methods which give reproducible results under controlled conditions.

Ayers *et al.* (1) successfully infected lespedeza plants with the wilt bacterium by three different methods of inoculation, all of which involved wounding of the host. The first method involved pricking the leaves with a needle dipped in a bacterial suspension. The second involved severing the roots of seedlings while they were immersed in a bacterial suspension. In the third, young leaflets were severed and the cut surface dipped in a bacterial suspension. They also attempted, with little success, to obtain infection by atomizing or swabbing the leaves of older plants with a bacterial suspension.

Wilsie and Hughes (7) found the needle-pricking technique to be

unsatisfactory as a means of evaluating selections of lespedeza for resistance to bacterial wilt. With their root inoculation technique, they obtained results similar to those obtained naturally in the field.

Hanson (9) was successful in infecting lespedeza plants by clipping 2 inches from the tops when they were 8 to 10 inches tall and dipping the cut ends in a bacterial suspension. Wilsie and Hughes (7), however, concluded that this method was so severe that different degrees of wilt resistance would be difficult to detect among lespedeza strains. Helm and McHoney (8) were unsuccessful in obtaining uniform wilt infections when they clipped lespedeza plants about 6 inches above the ground with shears just prior to applying the inoculum as a spray.

In lespedeza, the reactions of different strains to artificial inoculation do not fall into well-defined classes. Instead, a gradation is found from one class to another. When this condition exists, Brink *et al.* (2) stress the necessity of designating arbitrary class limits broad enough so that borderline cases will constitute only a small part of the total.

Wilsie and Hughes (7) used what they termed a "bacterial wilt index" to evaluate the relative resistance of lespedeza strains and selections to bacterial wilt. Their index was based on a relative scale which ranged from one to nine, with the lower numbers indicating a lesser degree and the higher numbers a greater degree of infection. A similar index was used by Ayers *et al.* (1), except that their scale included only five classes as follows: zero, no infection; one, very light; two, light; three, moderate; and four, severe infection. The wilt index used by Hanson (9) also included five arbitrary classes based on the severity of the disease as follows: none, trace, slight, moderate, and severe. Baldridge (11) estimated the percentage of infected leaves and classified the various strains and selections on that basis.

MATERIALS AND METHODS

Source and Concentration of the Inoculum: The causal organism was isolated in pure culture from diseased plants of the Early Korean F. C. 19604 strain of annual lespedeza. A 1-inch section of stem (Figure 2) was surface sterilized for 2 minutes in a calcium hypochlorite solution ("B-K" powder, 5 percent solution, filtered). After washing the stem section in sterile, distilled water to remove the sterilant, about ¼-inch was removed from each end with a sterile razor blade. The cut ends of the stem section then were streaked across agar plates with chemically sterilized forceps. Within 2 or 3 days an individual colony was selected and transferred to a tube of fresh nutrient agar. Subsequent transfers were made as needed.

All cultures of the pathogen were grown in test tubes on nutrient agar slants. Six-week transfers were necessary to keep the cultures alive. Virulent cultures were maintained by re-isolating the organism from diseased plants

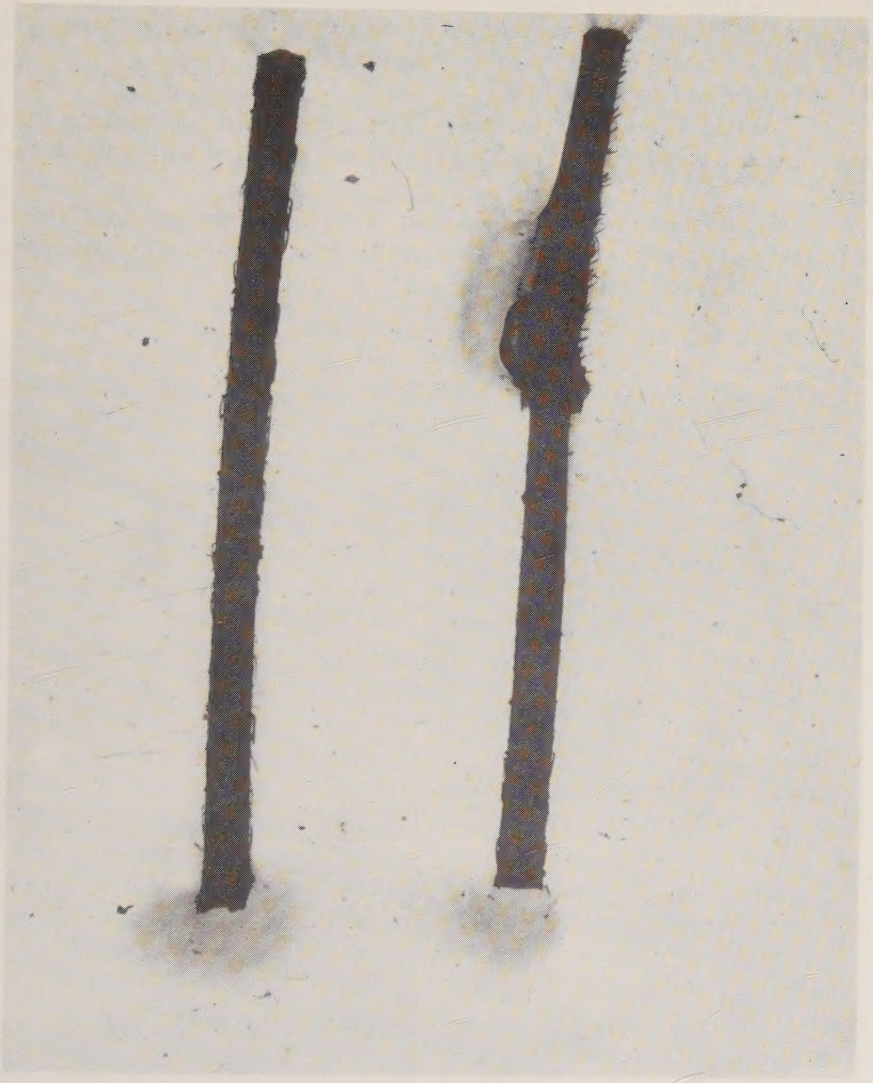

Fig. 2—A water mount of stem sections from diseased lespedeza plants showing the characteristic bacterial extrusions (streaming) from vascular bundles at the cut ends, and from a lesion on the stem at right, x 20.

of the F. C. 19604 strain at least once each year. The pathogenicity of each new isolate was compared with that of each preceding isolate so that the different isolates used would be similar in virulence. No difference in virulence was discernible.

A standardized concentration of inoculum was prepared by suspending

the growth from a two-week-old culture in 15 c.c. of sterile, distilled water just prior to its use. One culture per 15 c.c. of water was arbitrarily designated as a "standard" concentration or suspension.

Methods of Inoculation:

1. Cotyledon immersion method—With the seedlings immersed in the inoculum, the distal third of each cotyledon was removed with a sterile razor blade.

2. Root tip immersion method—About ¼-inch was removed from the tips of the primary roots with a sterile razor blade or scissors while the plants were immersed in inoculum (Figure 3A).

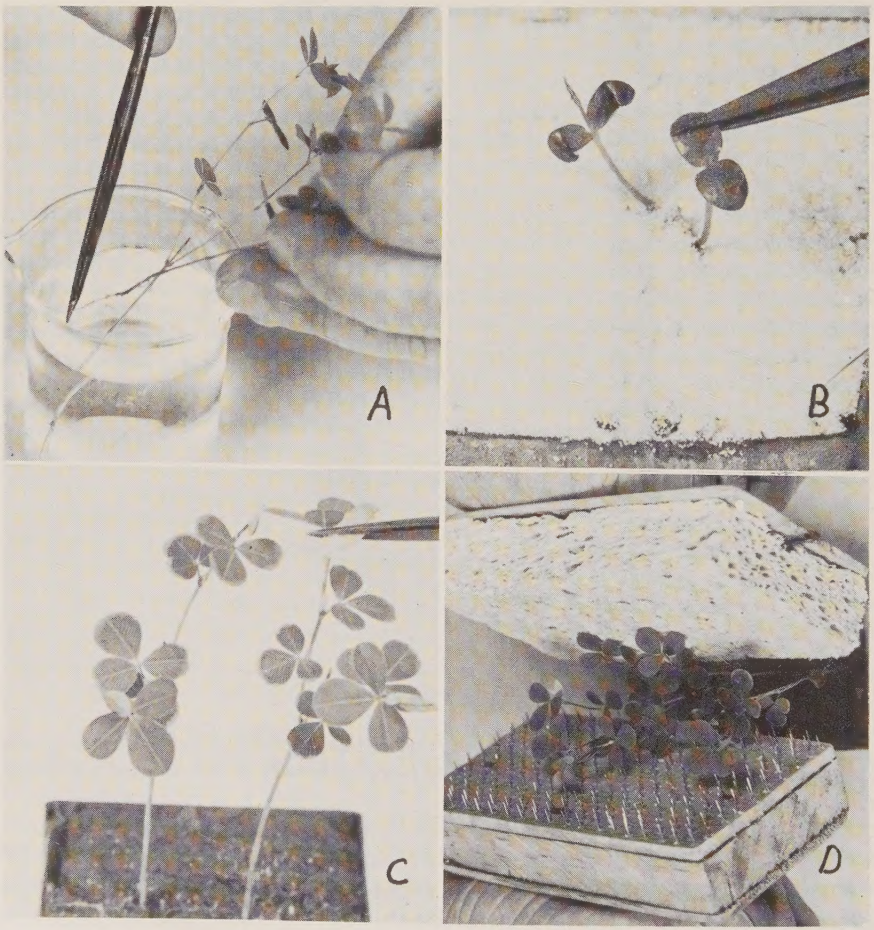

Fig. 3—Four of the inoculation wounding techniques used in this study: (A) Clipping immersed roots; (B) pinching cotyledons; (C) clipping growing points; (D) pricking with pins.

3. *Cotyledon and leaf clipping method*—Either the tips of cotyledons or the tips of trifoliate leaves were removed with a pair of fine-pointed scissors, which were dipped frequently in the inoculum.

4. *Cotyledon pinching method*—The middle of each cotyledon was pinched severely with forceps moistened with inoculum (Figure 3B).

5. *Growing point method*—The growing point and top trifoliate leaf petiole of each main branch were removed with scissors moistened with the inoculum (Figure 3C).

6. *Immersed stem clipping method*—With the foliage of plants immersed in the inoculum, about 2 inches was clipped from the tops with scissors.

7. *Non-immersed stem clipping method*—About 2 inches was clipped from the plant tops with scissors; the cut ends then were sprayed with the inoculum or were immersed in it for a few seconds.

8. *Sprayed-foliage clipping method*—The plant foliage was subjected to a fine spray of the inoculum and at the same time the plants were clipped 4 inches above the ground. The foliage and the shears were moistened with the spray throughout the process.

9. *Multiple pin method*—A sponge saturated with the inoculum was used to moisten a multiple pin inoculator which pricked a large number of leaflets simultaneously (Figure 3D).

Stages of Plant Development when Inoculations Were Made: Plants were inoculated at three stages of development. Seedlings with fully developed cotyledons and with the juvenile leaves (first pair of leaves above the cotyledons) just starting to unfold represented the youngest stage. Plants approximately 2½ inches tall, with the first trifoliate leaves unfolded, represented the intermediate stage. Plants 4 to 5 inches tall represented the more mature stage.

Strains of Lespedeza Used in the Study: The 10 strains which were used for the inoculation studies varied widely in date of maturity and in resistance to bacterial wilt under natural field conditions. One or more named strains was included in each test to provide a standard of comparison for evaluating the performance of the other strains.

Six of the 10 strains were of Korean *(L. stipulacea)* origin: F.C.19604, Iowa 6, Korean, Rowan, Climax, and Sel. 29a. Four of the 10 strains were of common *(L. striata)* origin: F.C.31057-5, F.C.31852, Kobe, and Sel. 22a.

In resistance to wilt the strains may be classified as follows: F.C.19604, highly susceptible; Iowa 6, Rowan, and Korean, susceptible; F.C.31057-5, F.C.31852, Climax, and Kobe moderately resistant; Sel. 29a and Sel. 22a, resistant. Sel. 29a and Sel. 22a were the most promising lines that had been selected prior to the inoculation studies. Sel. 29a was selected from Climax, and Sel. 22a from F.C.098171.

Evaluation of Wilt Resistance: Two methods of evaluating the wilt resistance of individual strains were used. One method was based on the per-

centage of inoculated plants in each strain that developed symptoms beyond the point of inoculation. The other method was based on the difference in weight of forage samples of inoculated and non-inoculated plants of the same strain. The reduction in yield of each strain due to wilt was computed in percent and used as the basis for rating the wilt resistance of the different strains.

Temperature, Light, and Soil Used in the Greenhouse Experiments: The greenhouse temperature fluctuated between 75° and 90° F, with the higher temperatures prevailing in the daytime on warm sunny days and the lower temperatures at night.

To prevent lespedeza plants from producing seed prematurely during the winter months, the normal day was extended 4 or 5 hours with 1000-watt lamps placed 3 feet above the plants.

Putnam silt loam soil was used in the greenhouse tests. Screened soil was placed either in 6-inch pots or in wooden flats containing seventy 2-inch by 2-inch metal bands.

Location, Plot Design, and Harvesting Methods Used in Field Investigations: The first field inoculation studies were made in 1951 and 1952 on a restricted area. In 1953 the experiments were moved to the University South Farms, Columbia, Mo., where a larger, more uniform area was available.

A split-plot design, which aided in the isolation of the treatments, was used. This arrangement permitted statistical comparison of the relative wilt resistance of strains and provided isolation of inoculation treatments, thereby decreasing the natural spread of wilt from one treatment to another by vectors such as insects or rainstorms.

The test plot was divided into main blocks 5 feet wide and 20 feet long, providing one main block for each inoculation treatment. The experimental strains were randomized within each main block and planted in 5-foot rows spaced 2 feet apart. Forage from a 2-foot section was harvested from the center of each 5-foot row for each treatment. The entire experiment was replicated four times.

Histological Methods: Four strains of annual lespedeza were selected for the histological study on the basis of their relative resistance to bacterial wilt as follows: F.C.19604, highly susceptible; Standard Korean, susceptible; Climax, moderately resistant; and Sel. 22a, resistant.

Each strain was grown under supplemented and unsupplemented winter light. Separate groups of plants from each strain were inoculated at two stages of development for each light condition. The first group of plants was inoculated when the juvenile leaves were half unfolded, and the second group when the plants were 4 inches tall.

Slides were prepared from stem sections taken just below the cotyledons on the younger plants, and just below the second node under the point of

Fig. 4—Lespedeza plants and leaflets infected with bacterial wilt. (A) Symptoms of water-soaking and curling of the leaflets (indicated by arrows). (B) Disease symptoms in two stages of development. Leaflets 1, 2, and 3, completely wilted, desiccated, and curled; leaflets 4, 5, and 6, just starting to water-soak at their tips; leaflets 7 and 8, healthy.

inoculation on the more mature plants. To demonstrate the presence of the organism within the leaves, slides were prepared from diseased F.C.19604 leaflets that were just beginning to show the characteristic water-soaking at the tips (Figure 4B). To show the distribution of the causal organism

within the fruit, slides were prepared from slightly immature seeds obtained from badly diseased F.C.19604 plants. Between 10 and 20 slides were prepared from plants within each treatment for each strain.

All plant material was killed and fixed in F.A.A. The younger plants were killed and fixed 7 days after inoculation, while the more mature plants were killed and fixed 14 days after inoculation. All specimens were dehydrated by the Dioxane method and subsequently embedded in paraffin prior to sectioning. The slides were stained by the Safranin-fast green technique.

Diagrams were prepared to show the distribution and spread of the causal bacterium within the host tissues from the point of inoculation. Several plants, which were selected at random from each of five strains (F.C.-19604, Korean, Climax, Sel. 29a, and Sel. 22a), were diagramed from 5 to 7 weeks after inoculation. The distribution of the organism was determined by bacterial extrusions from stem sections in water mounts.

RESULTS

Preliminary Investigations

The main objective was to study conditions favoring epiphytotics of bacterial wilt in annual lespedeza.

The Need for Wounding: The first experiment was made to determine whether wounds were necessary for the causal organism to enter and infect the host. Seeds of the F.C.19604 strain were germinated on moistened paper toweling in petri dishes. When the seedlings were ¾-inch long, they were transplanted to bands of soil.

Each treatment was comprised of three bands of five plants each. One treatment consisted of cutting off ⅓ of each cotyledon while the seedlings were immersed in a bacterial suspension (inoculum). The check plants were treated in the same manner except that the inoculum was replaced with sterile, distilled water. A second treatment consisted of transplanting the seedlings directly to bands of soil from the petri dishes without injuring them. Following transplanting, 10 c.c. of inoculum was poured over the foliage and around the roots of the seedlings. For the check treatment an equal quantity of sterile, distilled water was used instead of the inoculum.

Seven days after inoculation, all 15 of the plants which were inoculated by cutting off part of the cotyledons under a bacterial suspension were dead (Table 1). Only one of the 15 check plants showed symptoms of wilt. Where the inoculum was poured over the foliage and around the roots of the seedlings, only 2 of the 15 plants showed symptoms of wilt.

These results show that while a small amount of infection occurred without artificial wounding, such wounding was necessary for epiphytotics of the disease. It is suggested that the small amount of natural infection obtained in this and later greenhouse experiments arose from bacteria being

TABLE 1 -- WOUNDING AS RELATED TO INFECTION OF LESPEDEZA SEEDLINGS

Treatment	Seven Days After Inoculation	
	No. Plants Diseased	No. Plants Healthy
Cotyledons cut under bacterial suspension	15	0
Cotyledons cut under sterile water (check)	1	14
Bacterial suspension poured over foliage and around roots	2	13
Sterile water poured over foliage and around roots (check)	1	14

present in the seed hull and entering the plant through natural injury, usually to the cotyledons. In some instances there was opportunity during watering for splashing to spread the disease to check plants.

The need for wounding was demonstrated further in a second experiment (Table 2). Unhulled F.C.19604 seeds were germinated in petri dishes

TABLE 2 -- WOUNDING AS RELATED TO INFECTION OF GERMINATING SEEDS

Treatment	Seven Days After Transplantation	
	No. Seedlings Diseased	No. Seedlings Healthy
Germinated with bacterial suspension	0	15
Germinated with sterile water	1	14
Germinated with bacterial suspension followed by injury	15	0
Germinated with sterile water followed by injury	1	14

on paper toweling moistened with wilt inoculum. An equal number of seeds was germinated on paper toweling moistened with sterile, distilled water. Fifteen of the seedlings which germinated in the bacterial suspension were transferred uninjured to three bands of soil, five to each band. An equal number were injured before transplanting by placing on toweling saturated with inoculum and severing the cotyledons with a razor blade. The 30 check plants were handled similarly except that sterile water replaced the wilt inoculum.

In other tests inoculation by spraying with an atomizer did not produce the disease.

Primary Infection: In the field, wilt infected lespedeza plants first appear early in June. The infection starts in a few scattered plants and as the season progresses it may spread rapidly, depending on environmental conditions.

To study primary infection an experiment was performed in the green-

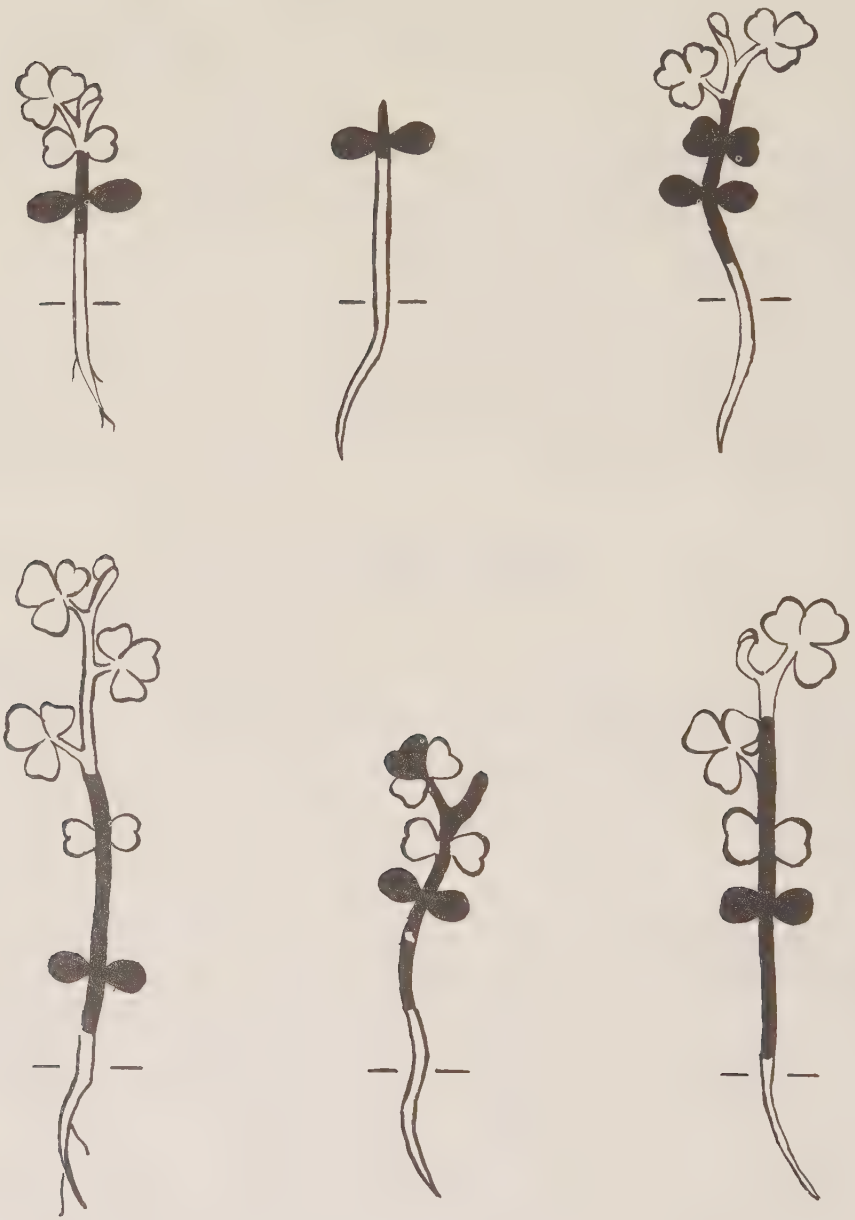

Fig. 5—Seedlings of strain F. C. 19604 showing the location, internally, (darkened areas) of the bacterial wilt organism in naturally infected plants. The diagrams were made as soon as external symptoms of wilt were observed.

house using a susceptible strain of lespedeza, F.C.19604. Seedings were made in flats of unsterilized soil in January, February, and March, 1953. The stand in each monthly seeding was thinned to 700 plants. The seedlings then were observed for wilt symptoms for a period of 30 days. When a plant was suspected of having wilt, it was sectioned and examined microscopically for the presence of the wilt bacterium (Figure 2). A diagram was prepared for each diseased plant to show the location of the organism within the plant (Figure 5).

Of the 2,100 plants observed, 34 (1.6 percent) were infected with bacterial wilt from natural infections (Table 3). In all of the diseased seedlings

TABLE 3.-- NUMBER AND PERCENT OF F.C.19604 LESPEDEZA SEEDLINGS INFECTED WITH BACTERIAL WILT FROM NATURAL INFECTIONS WHEN GROWN IN UNSTERILIZED GREENHOUSE SOIL

Month of Seeding	Number of Plants Under Observation	Infection	Percent Infected
January	700	14	2.00
February	700	12	1.71
March	700	8	1.14
Totals	2100	34	
Average			1.62

the vascular bundles in the cotyledons contained bacterial masses. Only a few juvenile leaves were found to contain infections. At the time external wilt symptoms first appeared, no plant contained bacterial masses below the crown, although several days later a few plants did contain the organism below that point. The cotyledons seemed to be the point of entry for bacteria in the primary infection.

Overwintering of the Wilt Organism: The parasite seems to overwinter in seed and diseased plant debris. The usual farm practice of letting lespedeza reseed and volunteer year after year probably encourages a build-up of the disease.

An experiment was performed to determine the possibility of the pathogen overwintering on the seed or in the hulls. Unhulled F.C.19604 seed harvested the previous year from diseased plants was soaked in 25 c.c. of sterile distilled water. After soaking 4 hours the suspension was filtered and the filtrate was used to inoculate 20 F.C.19604 seedlings by the growing point method of inoculation (Figure 3). An equal number were inoculated with sterile distilled water. Six days after inoculation, 17 of the 20 plants inoculated with the filtrate from soaked seed showed symptoms of wilt, while all of the check plants appeared healthy. The pathogen was re-isolated in pure culture from the diseased plants. Thus it appeared that unhulled seed could harbor the organism in a virulent condition from one season to the next.

Seed from diseased plants produced only a small percentage of diseased

seedlings. This suggested that the pathogen was harbored on the outside of the seed or in the hulls rather than on the inside of the seed. A study was made to determine in what part of the unhulled seed the pathogen was carried. Inoculum was prepared from diseased, unhulled seed of F.C.19604 plants by three methods: (1) Two c.c. of unhulled seed were soaked for 4 hours in sterile distilled water and filtered. The filtrate was used as the inoculum. (2) Two c.c. of hulled seed were surface sterilized in a 10 percent solution of calcium hypochlorite for 5 minutes, rinsed carefully in sterile distilled water, scarified, and then soaked overnight in 25 c.c. of sterile distilled water. After soaking overnight the seeds were filtered and the filtrate used as the inoculum. At the time of filtering the young radicles were ⅛-inch to ¼-inch long. Since the majority of the seed coats were ruptured during germination, bacteria within the seed coat could diffuse out into the water in which the seeds were soaking. (3) Two c.c. of hulls without the seeds were soaked for 4 hours in 25 c.c. of sterile distilled water and filtered. The filtrate was used as the inoculum.

Separate groups of F.C.19604 seedlings were inoculated when 2 inches tall with each of the three filtrates. A check group was inoculated with sterile distilled water. All inoculations were made with the growing point method. The number of plants with wilt was determined 21 days after inoculation.

Table 4 shows that the filtrates derived from hulls and from seeds plus the hulls, incited many seedlings (50.0 and 65.2 percent, respectively) to

TABLE 4 -- RESULTS OF INOCULATING F.C.19604 SEEDLINGS WITH FILTRATES OBTAINED FROM SEED OF DISEASED F.C.19604 PLANTS

Source of Filtrate	Number of Plants	
	Inoculated (1)	With Wilt
Hulls and seed	46	30
Hulls only	40	20
Seed only	154 (2)	5
Sterile water (check)	32	3

(1) Plants were inoculated with the growing point method.

(2) A large number of plants were used for this treatment to permit a comparison with the amount of wilt found previously (Table 3) from natural causes in the greenhouse.

develop the disease. Filtrate derived from hulled seed which had been surface sterilized did not increase the amount of wilt above that of the check treatment. The wilt organism was carried in the hull, probably in the vascular elements, but apparently was not carried in other parts of the seed. Small pieces of stems found in commercial seed also may harbor the pathogen.

The possibility of the parasite overwintering in plant debris was studied, using residues of diseased plants. Residues were collected in the winter and spring of 1953 from three strains: F.C.19604, Iowa 6 and Standard

Korean, all of which had been severely damaged by wilt in the field. Field samples were collected at monthly intervals from each strain and were taken to the laboratory for further study, beginning in January and ending in April (Figure 6).

In addition to testing for the presence of the bacterium (streaming), the virulence of the organism was determined. Stubble specimens were presumed to harbor virulent bacteria if a water filtrate derived from the stem sections produced the disease in F.C.19604 seedlings while check plants remained healthy. Virulent bacteria were present in the stems of all three strains collected at all dates.

Germination of Seed from Diseased Plants: To test the effect of possible bacteria harbored on the seed or in the hulls upon seed germination, the germination of seed from diseased plants was compared with that of seed from healthy plants. The seeds were germinated in petri dishes and germination counts were made after 5 days. Five replications of 100 seeds each

Fig. 6—Lespedeza stubble collected in March that contained the bacterial wilt organism in a virulent condition.

were used for each treatment. Table 5 shows that seed from diseased plants was not inferior in germination to that from healthy plants. However, under field conditions it is possible for the bacteria carried by the seed and plant residues to enter through wounds and infect emerging seedlings. Primary infection probably occurs in this manner in lespedeza fields.

TABLE 5 -- GERMINATION OF SEED FROM DISEASED AND HEALTHY PLANTS

	Percent Germination	
Replication	Seed From Diseased Plants	Seed From Healthy Plants
1	75	61
2	72	72
3	70	63
4	73	61
5	78	66
Average	73.6 (1)	64.6

(1) Significant at the .05 level.

Secondary Spread: In June wilt normally appears in only a few lespedeza plants scattered throughout the field, yet later in the season it may reach epiphytotic proportions, particularly in grazed or clipped stands. This increase as the season progresses probably represents a secondary spread of the organism from diseased to healthy plants.

A simple test was used to show the possibility of livestock spreading the disease through grazing from diseased to healthy plants. Foliage of diseased plants was taken into the mouth of the experimenter in a manner to simulate grazing. With the diseased material held in the mouth, 20 healthy plants were bitten off at the growing points. An equal number of check plants were treated similarly, except that the pathogen was excluded from the mouth. The treatments were made June 16 when lespedeza was 6 inches tall. After 8 days, half of the inoculated plants showed extensive wilt symptoms while the check plants remained substantially free of symptoms.

Field observations indicated that grasshoppers were spreading the disease to healthy plants. On many recently infected plants, leaves had been partially eaten by these insects, and the injured parts appeared to be points of origin of infection. In nearly every case a partially eaten leaf was adjacent to and connected with the area infected with wilt as determined by laboratory study (Figure 7).

The ability of grasshoppers to spread the disease was demonstrated on plants of strain F. C. 19604. Seed was germinated in pots of soil and grown for 2 weeks in cages to exclude grasshoppers. On August 4 half of the plants were inoculated with wilt and placed in a separate cage into which grasshoppers were introduced August 6. After feeding on the infected plants 2 days the grasshoppers were transferred to the cage of healthy plants, where they were allowed to feed 4 days and then were removed. By August 21,

Fig. 7—Diagram of a typical plant indicating the leaflets which had been partially eaten by insects and the areas within the plant where the wilt organism was found. Arrows indicate leaflets which were partially eaten by insects, and shaded areas show where the wilt organism was found.

fifty-one percent of the plants fed on by grasshoppers showed severe infection while check plants remained free of wilt.

On several occasions an increase in wilt was observed soon after mowing or clipping a stand of lespedeza. For example, in the 1952 lespedeza strain test only a few plants in susceptible varieties (F. C. 19604, Early Hy-

brid, Korean, and Iowa 6) showed symptoms of wilt before harvesting. After harvesting for hay the number of infected plants increased to many times the original number.

The spread of the disease by clipping was demonstrated experimentally. Seed of strain F. C. 19604 was planted in flats June 28. The plants were inoculated July 3 by placing the stems of diseased plants against the cotyledons of healthy seedlings and cutting with moistened scissors. Check plants were treated similarly except that healthy stems replaced diseased stems against the cotyledons. Table 6 shows that this method effectively spread the disease.

TABLE 6 -- THE SPREAD OF BACTERIAL WILT FROM DISEASED STEMS TO HEALTHY PLANTS BY CLIPPING

Treatment	Number of Plants Inoculated July 3	Number of Diseased Plants July 11	Number of Diseased Plants July 20
Diseased stems and healthy cotyledons cut simultaneously	40	21	40
Healthy stems and healthy cotyledons cut simultaneously	20	0	0

In another test it was shown that scissors moistened with water spread the disease most effectively (Table 7). Seedlings of strain F. C. 19604 were inoculated by the following methods: (1) Diseased stems were soaked in sterile water to obtain a bacterial suspension and scissors dipped in this suspension were used to cut off ⅓ of each cotyledon; (2) diseased stems were placed against the cotyledons of healthy seedlings and cut with dry scissors; (3) diseased stems were placed against the cotyledons and cut with moistened scissors; (4) healthy stems were placed against the cotyledons and cut with moistened scissors. Clipping with moistened scissors was more effective than with dry scissors in spreading the disease.

TABLE 7 -- THE EFFECT OF USING MOISTENED SCISSORS TO SPREAD WILT FROM DISEASED STEMS TO HEALTHY PLANTS BY CLIPPING

Inoculation Treatment	Number of Plants Inoculated July 16	Number of Diseased Plants July 23	Number of Diseased Plants July 25
Healthy cotyledons cut with scissors dipped in filtrate from diseased plants	20	17	19
Healthy cotyledons and diseased stems cut simultaneously with wet scissors	20	16	20
Healthy cotyledons and diseased stems cut simultaneously with dry scissors	20	3	4
Healthy cotyledons and healthy stems cut simultaneously with wet scissors (check)	20	0	1

Effect of Light: When lespedeza plants are grown in the greenhouse during the short days of winter, they do not develop normally. They tend

Fig. 8—The wilt resistance of two selected lines relative to that of the original parental lines when grown under the long days of summer: (A) Selection 29a (left) and Climax (right) from which 29a was selected. (B) Selection 22a (left) and P. I. 098171 (right) from which 22a was selected. Photographed July 23, 1951.

to be spindly and to have weak stems. In the seedling stage they are attacked readily by damping-off fungi. It was believed that lespedeza might be more susceptible to bacterial wilt under winter light than under light conditions at other times of the year. Evidence to support this belief was obtained during the screening of strains and selections for wilt resistance.

During the first screening in April six plants were found that were highly resistant. These selected lines survived three successive inoculations at 3-week intervals, the last two made to eliminate escapes.

Seed from these selections, along with seed from their parental lines, were scarified and planted in bands of soil on July 9. The resistant lines and their parental lines were inoculated on July 14 with the cotyledon pinching method. To help avoid escapes, all surviving plants were inoculated the second time on July 28 by clipping each leaflet. Observations made on August 10 showed that five of the six selections were maintaining good resistance (Figure 8).

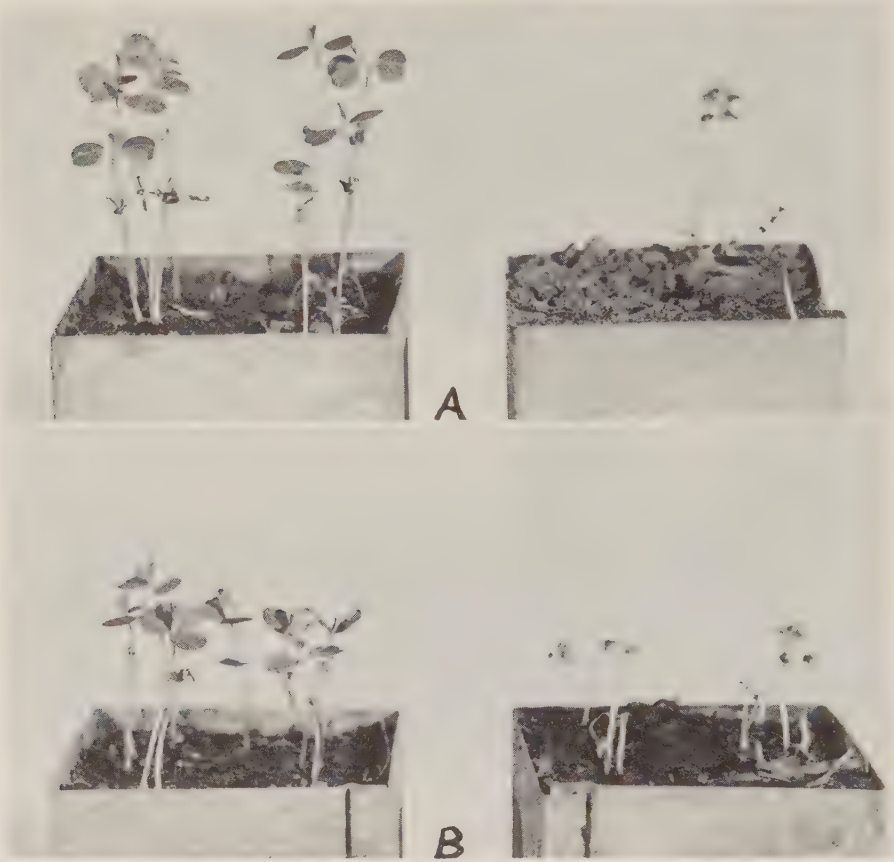

Fig. 9—The wilt resistance of two selected lines relative to that of the original parental lines when grown under the short days of winter without supplemental light: (A) Selection 29a (left) and Climax (right) from which 29a was selected. (B) Selection 22a (left) and P. I. 098171 (right) from which 22a was selected. Photographed December 27, 1951.

Seed from the resistant lines and their parental lines was harvested and a second generation test was started on November 23. The plants were inoculated by the cotyledon pinching method on December 6, at which time they were spindly and otherwise showed effects of unfavorable light conditions. Damping-off was more prevalent than in the two previous tests. Results of this test were disappointing. By December 31 most of the plants were either dead or severely wilted, both in the resistant lines and in their parental lines, although the rate of killing was more rapid in the latter than in the former (Figure 9).

To explain these results another experiment was made in which light conditions were varied. Four replications of F. C. 19604, Iowa 6, Korean,

Rowan, Climax, F. C. 31852, Kobe, Sel. 29a, Sel. 22a, and a Korean selection were planted in flats on January 10. Two replications were placed under each of two light conditions, one being the natural day of less than 12 hours occurring at this season, and the other being the natural day supplemented with 5 hours of artificial light. Supplemental light was obtained by placing 1000-watt Mazda lamps 3 feet above the plants from 5 p.m. to 10 p.m. All of the other environmental conditions were kept as nearly the same as was possible. Damping-off again was most pronounced under the unsupplemented day, and the plants were spindly. The plants in both treatments were inoculated on January 25 by the leaf clipping method.

By March 21 most of the plants in all strains grown under unsupplemented light were either dead or severely wilted (Figure 10). Those grown under the supplemented light were much less severely affected and there was a much greater difference between strains. Selections 29a and 22a were the most resistant, while F. C. 19604 was the least resistant of the ten strains.

These and other results showed that plants grown under the short days and low light intensity of winter were more susceptible to wilt than plants grown under longer days and greater light intensity. Supplementing winter light with light from 1000-watt Mazda lamps increased the resistance to wilt.

Effect on Forage Yield: Wilt obviously had reduced the forage yields of lespedeza but experimental evidence as to the amount was lacking. An experiment was made in the greenhouse to determine the reduction, using a method of artificial inoculation that was believed to approximate heavy natural infection.

Two replications of three strains, F. C. 19604, Climax, and Kobe, were planted on February 27 in flats of soil. Half of the plants in each replication

Fig. 10—Comparison of the effects of bacterial wilt on ten strains of annual lespedeza grown under long and short photoperiods. The flat on the left received less than a 12 hour day and the flat on the right received a 17 hour day. Photographed March 31, 1952.

were inoculated on April 16 with the growing point method. On this date, F.C. 19604 was 5 inches tall and the other two strains were 4 inches tall. The other half of the plants were handled in a similar manner except that no inoculum was applied to the cut surfaces. Dry weights were taken May 28. In this experiment bacterial wilt reduced the forage yield of F. C. 19604 by 66.0 percent, that of Kobe by 49.5 percent, and that of Climax by 41.4 percent. These reductions were higher than expected for the Kobe and Climax strains. Under the conditions of this experiment, the two strains did not show as great resistance as they had shown in field variety tests. Observations indicated that reduction in forage yields took place in two ways: First, the inoculated plants were stunted in growth; second, they dropped a higher percentage of their leaves. Thus, the quality as well as the quantity of forage was lowered.

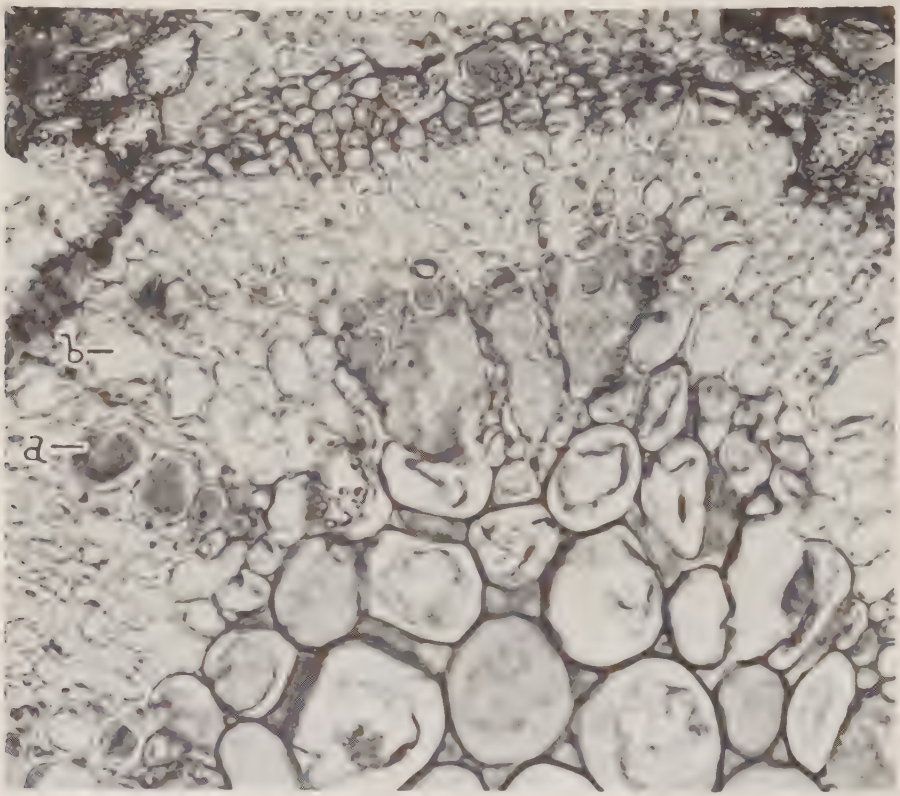

Fig. 11—Cross section of a diseased F. C. 19604 lespedeza stem, x550. Note that at this stage the bacterial masses are confined to the vascular bundles: (a) A xylem vessel clogged with bacteria; (b) a xylem vessel free of bacteria. The plant from which this specimen was taken was grown under a 15-hour day and was 4 inches tall when inoculated. This specimen was killed and fixed 14 days after inoculation.

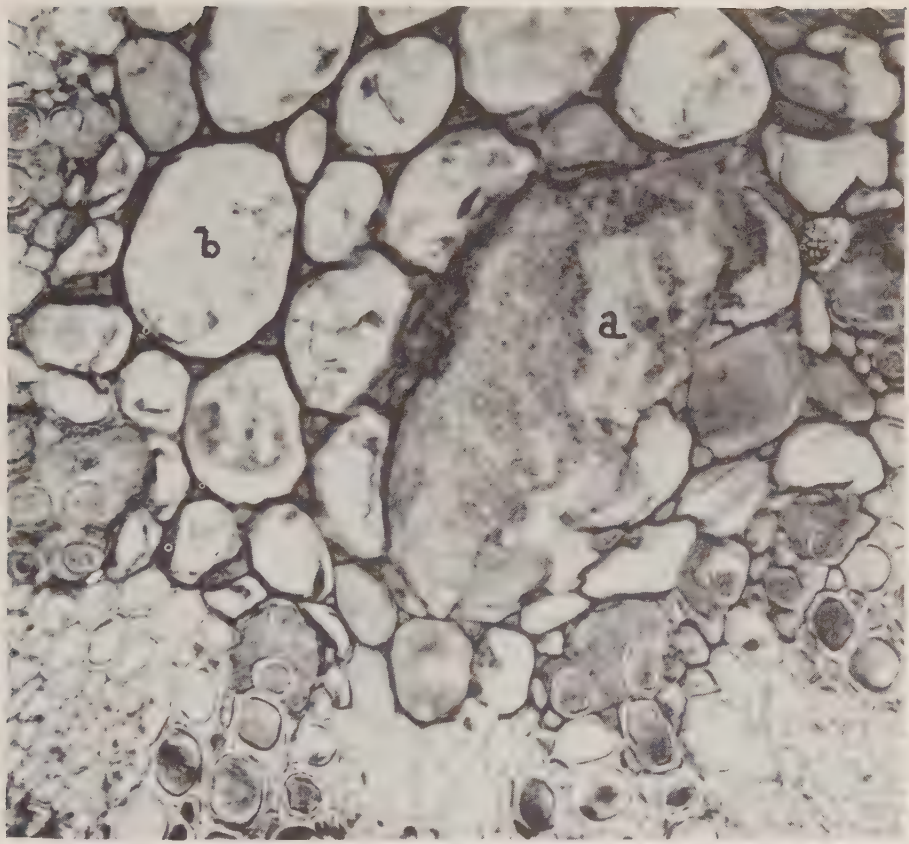

Fig. 12—Cross section of a diseased F. C. 19604 lespedeza stem showing the bacterial masses invading the pith cells after breaking from the vascular elements, x550: (a) Pith cell invaded by bacteria; (b) pith cell free of bacteria. The plant from which this specimen was taken was grown under a 15-hour day and was 4 inches tall when inoculated. The specimen was killed and fixed 6 weeks after inoculation.

Histological Studies

The objective was to investigate the spread and distribution of the bacteria in plants of different strains, inoculated at different stages of growth and grown under varying light conditions. The strains used were F. C. 19604, Korean, Climax, and Sel. 22a; the day lengths were 11 and 15 hours; the stages of growth were the cotyledonary stage and the 4-inch stage.

In the initial stage of infection following inoculation, the invading bacteria appeared to be confined to the xylem vessels (Figure 11). As the invasion progressed the cell walls of the xylem tissues were destroyed and the bacteria then were able to spread either inward toward the pith cells (Figure 12) or outward through the cortex cells (Figure 13). In some cases

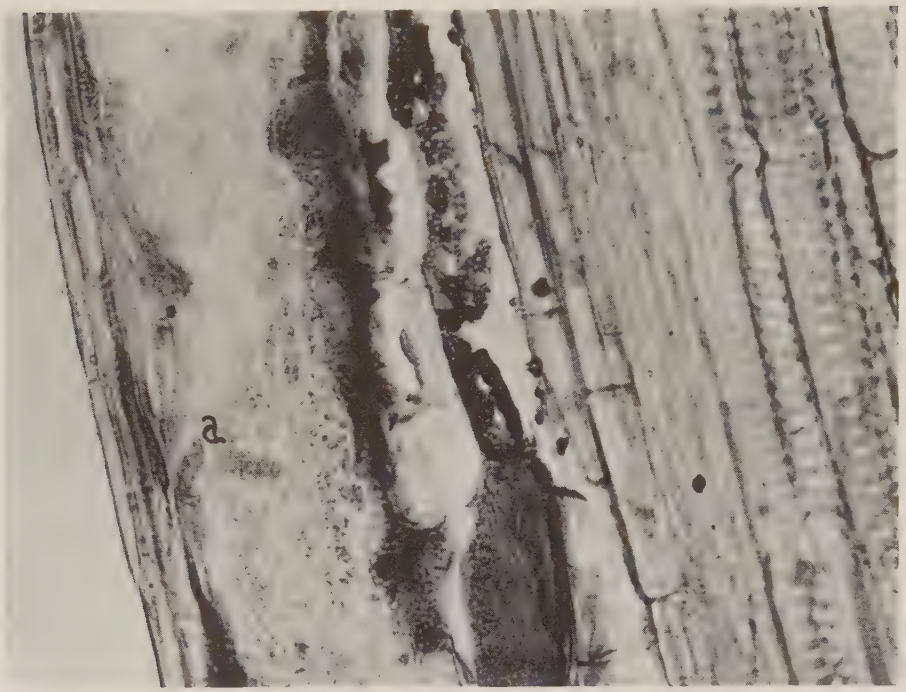

Fig. 13—Longitudinal section of a diseased F. C. 19604 lespedeza stem showing the bacterial masses breaking from the vascular bundles outward through the cortex, x550: (a) Cortex cells invaded by bacteria. The plant from which this specimen was taken was grown under an 11-hour day and was inoculated when it was 4 inches tall. The specimen was killed and fixed 6 weeks after inoculation.

the bacterial masses spread in both directions.

It was shown by two methods that the xylem vessels were broken down and eventually destroyed by the causal bacterium. First, xylem tissue breakdown was demonstrated by the Thiobarbituric Acid Aldehyde Test. When the thiobarbituric acid was applied to freehand sections of diseased stems, rather heavy concentrations of aldehydes were found in and near the infected vascular bundles. Only normal concentrations were associated with the vascular bundles which were free of bacteria. Second, slides (prepared by the paraffin method) of plant specimens in advanced stage of infection showed that all of the cell material in the xylem vessels was destroyed with the exception of the spirals (Figure 14).

The bacteria within infected leaflets appeared to be confined to the vascular bundles (Figure 15), except in the water soaked areas. In the water soaked areas the bacterial masses were distributed throughout the affected tissues. In some instances a bacterial exudate was found upon the surface of the water-soaked parts of the leaflets.

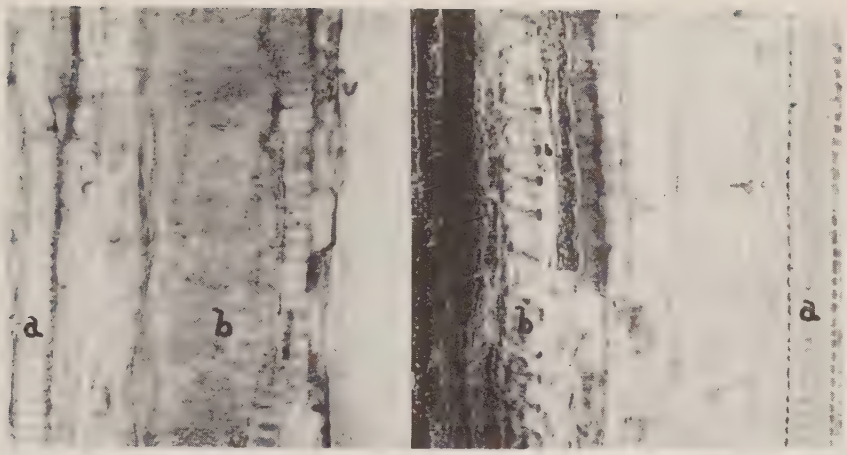

Fig. 14—Two longitudinal sections of diseased F. C. 19604 lespedeza stems showing the destruction of the xylem vessels. x440: (a) Xylem vessels free of and uninjured by bacteria; (b) xylem vessels destroyed by bacteria (note the presence of the free spirals).

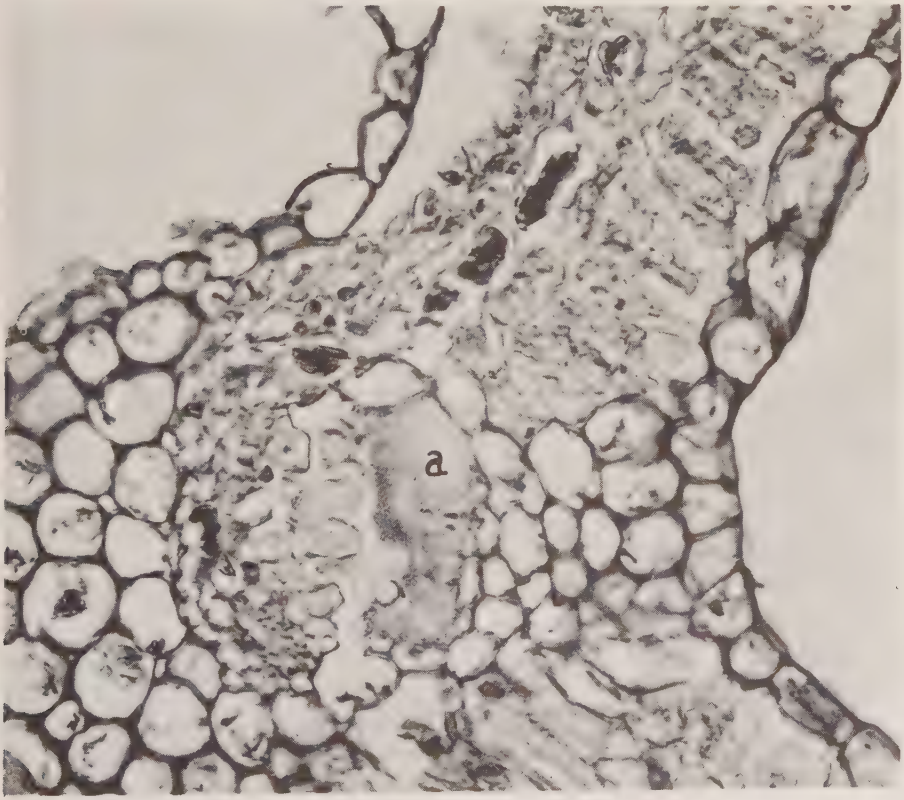

Fig. 15—Cross section of the central part of a diseased lespedeza leaflet taken from an area that was not water-soaked, x550: (a) Xylem vessels clogged by bacteria.

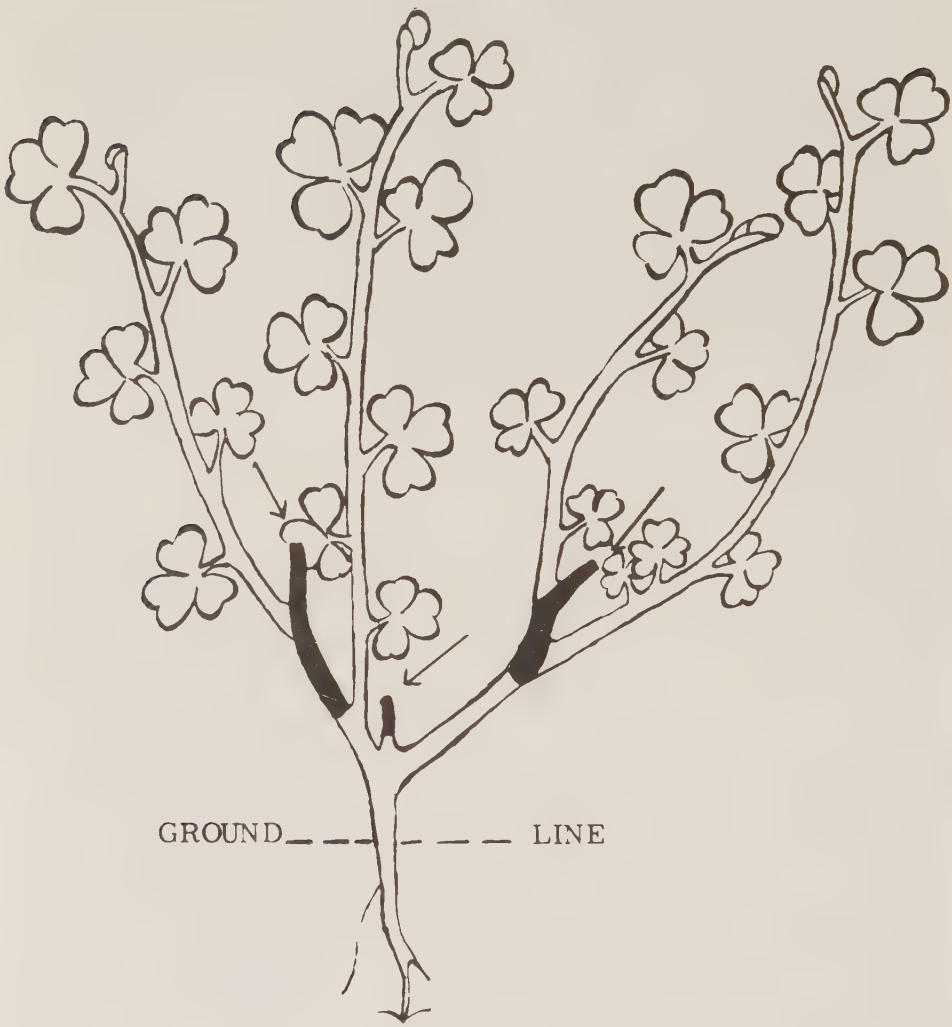

Fig. 16—A plant of Sel. 29a showing the limited spread of the wilt organism within the more resistant strains. The diagram was prepared 49 days after the plant was inoculated. Arrows indicate the points of inoculation and darkened areas show regions of infection.

Within the fruits the bacterial cells were found in the vascular tissues of the hull but were not found within the embryo or within the seed coat. Only a small number of seeds were investigated.

In plants inoculated in the cotyledonary stage, little difference was observed in cell wall development and cell clogging as related to light conditions. Some difference was found between strains. Plants in the cotyledonary stage contained fewer fully developed xylem vessels per cross section of stem

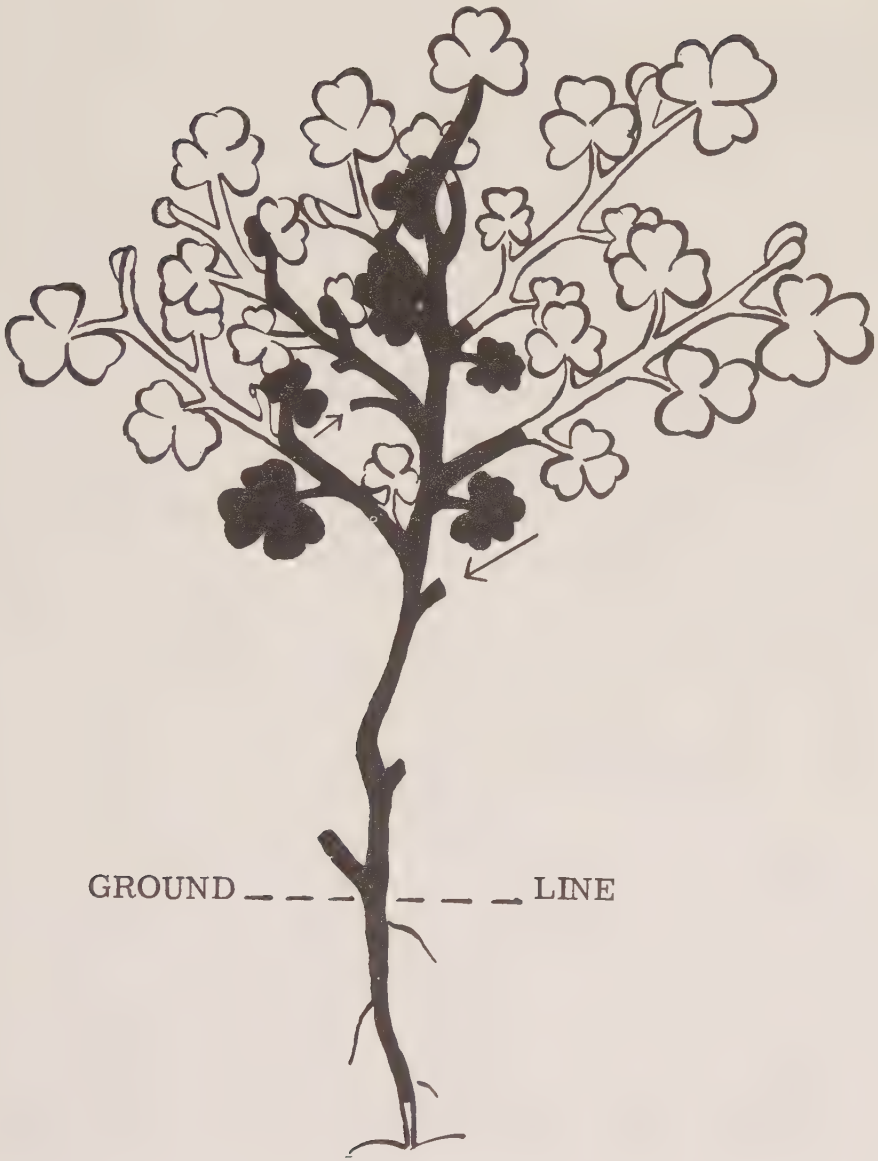

Fig. 17—A plant of F.C.19604 showing the extensive spread of the wilt organism within the more susceptible strains. The diagram was prepared 35 days after inoculation. Note that the disease has spread from the main stem into some of the side branches. Arrows indicate the points of inoculation and darkened areas show regions of infection.

than did plants 4 inches tall. A larger proportion of the xylem vessels was clogged and destroyed by the bacteria in the younger plants than in the more mature ones. Strain differences were not as great in the younger plants

as they were in the older ones, but even in the early stages of growth the vascular bundles of the more resistant strains were clogged less by the bacterial masses than were the more susceptible ones.

Greater differences between strains and between light treatments were found when inoculations were made at the older stage of growth. In this stage plants grown under unsupplemented light had thinner xylem cell walls than plants grown under supplemented light. The susceptible strains appeared to have thinner xylem cell walls than did the more resistant strains. Differences in cell wall thickness between light treatments appeared to be nearly as pronounced as the differences between strains.

It should be pointed out that the supplemental light increased not only the photoperiod but also the total illumination per day and the air and soil temperature while the lights were on.

Plant diagrams of artificially inoculated plants indicated that the spread of the organism within the host was more extensive in susceptible strains than it was in more resistant strains. With few exceptions the plants examined by this method showed the bacterial cells near the point of inoculation. In the more resistant strains (Figure 16), the organism was confined to a smaller area than it was in the susceptible strains (Figure 17). It appeared that the organism passed from the main stem into the side branches of susceptible strains more readily than it did in more resistant strains.

Investigation of Inoculation Methods in the Greenhouse

Root Versus Foliage Inoculation: Root inoculation was compared with foliage inoculation on three strains of lespedeza in two stages of growth. The experiment was begun February 20.

Three strains were grown in petri dishes until the plants were ¾-inch long, for the younger stage. The seedlings were removed, immersed in a standard bacterial suspension and the root tips or the cotyledons severed with a razor blade. After inoculation they were transferred to soil.

Three strains were grown in pots until 5 inches tall for the older growth stage. They were removed, immersed in a standard bacterial suspension, and inoculated by severing the root tips or the growing points of each foliage branch. After inoculation they were transferred to soil.

TABLE 8 -- COMPARISON OF ROOT WITH FOLIAGE INOCULATION OF LESPEDEZA IN TWO STAGES OF GROWTH

Stage of Growth	Percent of Plants Showing Wilt Symptoms	
	Root Inoculation	Foliage Inoculation
Cotyledonary stage	63	100
Five inches tall	70	93

Since the differences between strains were unreliable, the strains were combined in Table 8. Foliage inoculation was more severe than root inoculation in this experiment. Stage of growth influenced susceptibility, the disease developing rapidly in the younger stage with readings being taken after 17 days, and slowly in the older stage with readings being taken after 45 days. The younger stage was more susceptible.

Excessive labor requirement is an objection to root inoculation. Foliage inoculation can be made without transplanting and, as a result, it facilitates the handling of large populations.

Cotyledon Pinching Method: The cotyledon pinching method was devised as an easy, rapid method of foliage inoculation. It was compared with the cotyledon immersion method as to uniformity and severity of infection.

Seedlings of strain F.C.19604 were inoculated March 10 when 1-inch tall with the standard bacterial suspension.

Results are presented in Table 9. The disease was slower to develop with the pinching method but the outcome was substantially the same with the two methods.

TABLE 9 -- COMPARISON OF THE COTYLEDON PINCHING WITH THE COTYLEDON IMMERSION METHOD OF INOCULATING LESPEDEZA SEEDLINGS

Method of Inoculation	Percent of Plants Showing Wilt Symptoms		
	March 21	March 29	April 6
Cotyledon pinching	33.8	80.0	90.8
Cotyledon immersion	52.7	93.2	97.3

Stem Clipping Methods: Immersed stem clipping was compared with non-immersed stem clipping as a means of producing infection.

Strain F.C.19604 was planted in flats of soil on February 8. Inoculations were made with the standard bacterial suspension on March 30 when the plants were 5 inches tall.

Table 10 shows that the two methods were about equal in effect. The immersed stem clipping gave slightly greater infection than the non-immersed; however, the latter method required less labor and was much better adapted for mass inoculations.

TABLE 10 -- COMPARISON OF THE IMMERSED WITH THE NON-IMMERSED METHOD OF INOCULATING LESPEDEZA PLANTS

Method of Inoculation	Percent of Plants Showing Wilt Symptoms	
	April 20	May 7
Immersed stems	18	92
Non-immersed stems	20	80
Non-inoculated (check)	0	0

The Growing Point and Multiple Pin Methods: The growing point method consisted of cutting the growing point and top trifoliate leaf petiole of each main branch with scissors moistened with inoculum. This method worked well in the field giving reliable differentiation in resistance among lespedeza strains. It gave less reliable results in the greenhouse, probably due to the greater severity of infection.

It was believed that the multiple pin method would give a milder infection that would be more suitable for evaluating resistance under greenhouse conditions and give greater economy of labor. In making inoculations the inoculator piece containing the sponge was saturated with inoculum and held against the plants to be treated. The piece containing the pins was pressed firmly against the sponge and the operation repeated twice. The pins pierced the leaves of many plants, introducing the inoculum (Figure 18).

The growing point and multiple pin methods were compared on three strains, F.C.19604, Standard Korean, and Sel. 29a. The plants were grown in the greenhouse in flats using nine replications for each treatment. Inoculations using the standard bacterial suspension were made April 3 when the plants were 4 inches tall. Dry weights taken June 1 were used as the measure of wilt damage.

Fig. 18—Plants of three strains of lespedeza 19 days after inoculation by the multiple pin method. Left, F. C. 19604; center, Korean; right, Sel. 29a. Note the pin pricks in the lower leaves.

The multiple pin method gave somewhat better differentiation between strains than the growing point method (Table 11). The interaction of strains

TABLE 11 -- YIELDS OF THREE STRAINS OF LESPEDEZA UNDER TWO METHODS OF INOCULATION

Method of Inoculation	Variety Forage Yields in Grams Dry Matter			
	F.C.19604	Korean	Sel. 29a	Average[1]
Growing point	0.42	0.33	0.94	0.56
Multiple needle	1.41	1.54	2.62	1.86
Uninoculated	2.46	2.43	2.38	2.42
Average*	1.43	1.43	1.98	

[1] L.S.D. (.05): Varieties 0.12; methods 0.47.

and methods was significant at the 5 percent level. The percentage reduction in yields for the multiple needle and growing point methods, respectively, were: F.C.19604, 42.7 and 82.8; Korean, 36.6 and 86.4; Sel. 29a, 0.0 and 60.5.

Concentration of Bacteria and Stage of Plant Growth: In the histological studies it appeared that susceptibility to wilt was influenced by the stage of growth in which plants were inoculated. An experiment was made to test inoculation in three stages of growth: (1) The cotyledonary stage; (2) the first trifoliate leaf stage; (3) the 5-inch tall stage. Three concentrations of bacteria, standard, $^1/_5$-standard, and $^1/_{25}$-standard were used. Seven strains of lespedeza were tested, F.C.19604, Iowa 6, Korean, Rowan, Climax, F.C.31852, and Kobe.

Inoculations were made in all three growth stages on April 26. This was made possible by staggering the date of planting for each growth stage. The oldest growth stage was planted February 25, the medium stage March 28, and the youngest stage April 18. All growth stages were inoculated with the growing point method. The plants were moved out of the greenhouse soon after inoculation because of excessively high greenhouse temperatures.

Final readings were taken July 8, 74 days after inoculation. The results are presented in Table 12. Since the differences between strains were not

TABLE 12 -- INOCULATION OF LESPEDEZA IN THREE STAGES OF GROWTH WITH THREE CONCENTRATIONS OF THE WILT ORGANISM

Stage of Growth	Percent of Plants Showing Wilt Symptoms			
	Standard[1]	1/5 Standard[1]	1/25 Standard[1]	Sterile Water
Five inches tall	35.3	22.3	19.1	8.0
First trifoliate leaf	61.4	43.8	38.9	6.3
Cotyledonary	98.5	98.2	99.1	27.8

[1] A standard suspension was one 2-weeks-old test tube culture per 15 c.c. of sterile distilled water.

great, except for F.C.19604 which was heavily damaged, the strains were averaged together.

The data show that the plants were less susceptible to wilt in advanced stages of growth. Decreasing the concentration of bacteria in the inoculum decreased the amount of infection. The relatively high incidence of wilt on plants inoculated with sterile water is explained by the spread of the disease from diseased to healthy plants, probably by insects. The younger plants were affected most because they were in a relatively more susceptible stage of growth. The disease appeared on the check plants at a later date than on the inoculated plants.

A second experiment dealing with the same problems showed that significant differences in wilt reaction between a susceptible strain (F.C.19604) and a tolerant one (Sel. 29a) were not obtained in the young cotyledonary stage, but were obtained at later growth stages (Table 13).

TABLE 13 -- INOCULATION OF LESPEDEZA IN THREE STAGES OF GROWTH WITH DIFFERENT CONCENTRATIONS OF THE WILT ORGANISM

Stage of Growth	Concentration of bacteria[1]	Strain of Lespedeza	Percent of plants Dead 6 Weeks After Inoculation	
Cotyledons	S	F.C.19604	94[2]	100[3]
fully opened	S	Sel. 29a	88[2]	100[3]
	.01S	F.C.19604	36[2]	67[3]
	.01S	Sel. 29a	40[2]	80[3]
	.0001S	F.C.19604	7[2]	13[3]
	.0001S	Sel. 29a	10[2]	19[3]
Juvenile leaves	S	F.C.19604	44[4]	60[5]
fully opened	S	Sel. 29a	11[4]	21[5]
	.01S	F.C.19604	10[4]	15[5]
	.01S	Sel. 29a	2[4]	3[5]
Plants 4 inches tall	S	F.C.19604	20[6]	
	S	Sel. 29a	7[6]	
	.01S	F.C.19604	4[6]	
	.01S	Sel. 29a	1[6]	
Uninoculated controls	-	F.C.19604	4	
	-	Sel. 29a	1	

1/ S = standard suspension.
2/ One cotyledon inoculated by pinching.
3/ Both cotyledons inoculated by pinching.
4/ One juvenile leaf inoculated by clipping.
5/ Both juvenile leaves inoculated by clipping.
6/ Plants inoculated by cutting in half.

In the youngest stage both strains were largely eliminated when inoculated with the standard suspension. Reducing the concentration of bacteria in the inoculum reduced the damage from wilt and less severe wounding (one cotyledon) reduced the damage still more. There was no significant difference between strains at the 5 percent level under any of the conditions.

In the juvenile stage there was a significant difference between strains under all conditions.

In the 4-inch growth stage there was a significant difference between strains at the standard concentration but not at $^1/_{100}$-standard concentration.

The extremely dilute $^1/_{10\,000}$-standard concentration caused a slight reduction of stand in the youngest growth stage. The reduction was significant with inoculation in both cotyledons. The $^1/_{100}$-standard and the standard concentrations caused reductions that were significant or bordered on significance in the cotyledonary and juvenile stages.

In many cases heavier wounding significantly increased the stand losses when measured 6 weeks after inoculation. Infection, apparently, spread more rapidly in a given time when greater numbers of bacteria were introduced.

With advancing growth stages it required a greater concentration of bacteria in the inoculum to obtain a given reduction in stand in a given length of time.

The plants in the experiment above were grown in flats of soil in the greenhouse. Each treatment was replicated five times, and each replication contained 20 plants. The cotyledons were inoculated by pinching with tweezers dipped in inoculum, the juvenile leaves by clipping in half with scissors, and the 4-inch plants by cutting in half with scissors dipped in inoculum.

The experiment began March 26 and continued until June 12. Final readings were taken 6 weeks after each inoculation. This meant that each treatment was evaluated on the basis of the effect it produced during a period of 6 weeks and, had the experiment continued to the end of the growing season, the results might have been somewhat different. Since all plants were started at the same calendar date, the inoculation date was advanced for each growth stage. The changing temperature and light conditions in the greenhouse as the season advanced may have influenced the disease reactions of the different growth stages. High temperature and crowding of plants may have retarded plant growth and reduced wilt damage at later stages of inoculation. The results in general agree with those of the previous experiment.

Investigation of Field Inoculation Methods

1951 Results: A field experiment was started in the spring of 1951 to determine the effect of varying the concentration of bacteria in the inoculum. The strains used were F.C.19604, Climax, Korean, Kobe, and Rowan. These were planted April 26 in 6-foot rows spaced 3 inches apart. Each row was divided into four equal sections. One of the sections was inoculated with a double standard concentration of bacteria, one with a standard concentration, one with a one-half standard concentration, and the remaining section was left uninoculated as a check. All of the inoculations were made on June 8 with the sprayed-foliage clipping method, when the plants were 4 to 5 inches tall. This method of inoculation reduced the time required for making field inoculations.

On June 15, one week after inoculation, wilt symptoms were present on all inoculated plants. A count of infected plants was made on June 21 and the results are presented in Table 14. In general, the amount of infection in-

TABLE 14 -- INOCULATION OF LESPEDEZA WITH THREE CONCENTRATIONS OF THE WILT ORGANISM USING THE SPRAYED-FOLIAGE CLIPPING METHOD; FIELD STUDIES, 1951

Strain	Percent of Plants Showing Wilt Symptoms		
	2 Standard	Standard	1/2 Standard
F.C.19604	83.3	71.1	63.6
Climax	43.6	40.5	32.8
Korean	47.4	72.2	35.7
Kobe	53.3	30.0	30.8
Rowan	53.8	34.1	25.0

creased with increasing concentration of bacteria in the inoculum. Differences between strains were small and inconsistent, except for F.C.19604 which, invariably, was more heavily infected than the others.

1952 Results: The sprayed-foliage clipping method of inoculation was tested again in 1952 on F.C.19604, Korean, Kobe, Sel. 22a, Sel. 29a, and Climax. The plot was seeded on April 18, with each strain replicated three times in 6-foot rows spaced 12 inches apart. A 3-foot section of each row was inoculated on June 23 with the standard bacterial concentration and the remaining 3-foot section was left uninoculated as the check. The plants were from 6 to 9 inches tall at the time of inoculation.

Readings were made on July 30 to determine the percentage of plants with wilt symptoms. Forage samples were harvested on August 12 to determine the reduction in forage yields due to the disease. The results are presented in Table 15. Infection was not uniform within strains, and the results

TABLE 15 -- INOCULATION OF LESPEDEZA WITH THE SPRAYED-FOLIAGE CLIPPING METHOD; FIELD STUDIES, 1952

Strain	Percent of Plants Showing Wilt Symptoms	Percent Reduction of Forage Yields Due to Bacterial Wilt
F.C.19604	96.0	44.6
Korean	67.3	4.1
Climax	39.3	7.5
Sel. 29a	24.0	19.5
Kobe	42.0	13.6
Sel. 22a	11.0	8.5[1]

[1]The yield of the inoculated plants was 8.5 percent greater than that of the uninoculated plants.

were not in close agreement with those of the previous year. The relationship between the percentage of infected plants and the percentage reduction in forage yields was not as close as expected. If only the percentage of infected plants is determined following inoculation, a true picture of the wilt

damage may not be obtained. It would appear that the percentage reduction in forage yield is a better indication of the actual damage caused by wilt, since weight measurements take into account the damage resulting from stunting and from dropping of diseased leaves.

1953 Results: Previous greenhouse studies had shown that the severity of the disease was influenced by varying either the stage of growth when the plants were inoculated or the concentration of the bacteria in the inoculum. Using the greenhouse results as a guide, an experiment was made to determine the most suitable combination of the two variables for testing strains of lespedeza for wilt resistance in the field. The field experiment was planted in a replicated split-plot design with strains as sub-plots. The test included five main treatments. Two were inoculated when the first trifoliate leaves were fully formed, one with a standard bacterial concentration, and the other with a ⅕-standard concentration. Two other treatments were inoculated with the same concentrations after the plants had attained an average height of 4 inches. The fifth, or uninoculated block, was used as the check. All inoculations were made by the growing point method.

Seedings were made on April 21 and 24. Two treatments were inoculated on May 29 and 30 when the plants were in the younger growth stage. The three remaining treatments, two containing plants in the mature stage and the check treatment, were inoculated on June 23, 24, and 26.

The experiment was irrigated twice because of the extremely dry weather during the summer of 1953. An inch of water was applied each time, first on June 19, and again on July 27. The application dates were timely, since no rain fell within a week of either irrigation date.

All of the strains were harvested August 17, oven dried, and weighed.

In the analysis of variance, significant differences existed between inoculation treatments and between strains. Interaction between inoculation treatments and strains also was significant.

TABLE 16 -- EFFECT OF WILT INOCULATION TREATMENTS ON THE FORAGE YIELD OF LESPEDEZA; FIELD STUDIES, 1953

Inoculation Treatment[1]	Average Yield of Ten Strains in Tons Per Acre[2]
Uninoculated (check)	1.64
Small-S	1.12
Small-1/5S	1.29
Large-S	1.16
Large-1/5S	1.20

[1] Small-S = plants inoculated in the first trifoliate leaf stage with a standard bacterial suspension.
Small-1/5S = plants inoculated in the first trifoliate leaf stage with a 1/5-standard bacterial suspension.
Large-S = 4-inch tall plants inoculated with a standard bacterial suspension.
Large-1/5S = 4-inch tall plants inoculated with a 1/5-standard bacterial suspension.

[2] L.S.D. (.05) = 0.12 ton.

Considerable reduction in yield resulted from each inoculation treatment (Table 16). Differences between concentrations of bacteria in the inoculum were small and differences between the two stages of plant growth were insignificant. In the uninoculated treatment where wilt was absent, F.C.19604, Rowan, Iowa 6, and Korean were higher in yield than Sel. 29a (Table 17). In the inoculated treatments, Sel. 29a was invariably the highest yielding strain, and consistently outyielded Climax, the parent from which it originated.

TABLE 17 -- EFFECT OF WILT INOCULATION TREATMENTS ON THE FORAGE YIELD OF TEN LESPEDEZA STRAINS; FIELD STUDIES, 1953

	Yields in Tons Per Acre From Inoculation Treatments				
Strain	Uninoculated	Small-S	Small-1/5S	Large-S	Large-1/5S
F.C.19604	2.08	1.15	1.23	.99	1.10
Iowa 6	1.94	1.18	1.40	1.14	1.21
Korean	1.92	.95	1.15	1.06	1.01
Rowan	1.98	1.12	1.33	1.22	1.46
Sel. 29a	1.62	1.30	1.80	1.46	1.59
Climax	1.45	1.14	1.23	1.13	1.36
F.C.31057-5	1.36	1.02	1.22	1.16	1.18
F.C.31852	1.57	1.16	1.34	1.33	1.26
Kobe	1.51	1.15	1.23	1.18	1.19
Sel. 22a	.96	.99	.99	.92	.71[1]

L.S.D. (.05) between strains = 0.26 ton

[1]The low yield of Sel. 22a in this treatment was due to a poor stand.

The reduction in yield due to wilt ranged from 4.9 percent for Sel. 29a and 6.3 percent for Sel. 22a to 46.2 percent for F.C.19604 (Table 18, Figure 19). The average for all treatments and all strains was 27.6 percent. The proportionate reduction was less for Sel. 22a than for Sel. 29a, with one excep-

TABLE 18 -- PERCENTAGE REDUCTION IN FORAGE YIELDS RESULTING FROM FOUR INOCULATION TREATMENTS ON TEN STRAINS OF LESPEDEZA; FIELD STUDIES, 1953

	Percent Reduction in Yields From Four Inoculation Treatments				
Strain	Small-S	Small-1/5S	Large-S	Large-1/5S	Avg.
F.C.19604	44.7	40.9	52.4	47.1	46.2
Iowa 6	39.2	27.8	41.2	37.6	36.6
Korean	50.5	40.1	44.8	47.4	45.8
Rowan	43.4	32.8	38.4	26.3	35.4
Sel. 29a	19.7	11.1[1]	9.9	1.9	4.9
Climax	21.4	15.2	22.1	6.2	15.9
F.C.31057-5	23.5	10.3	14.7	13.3	15.4
F.C.31852	26.1	14.6	15.3	19.7	19.1
Kobe	23.8	18.5	21.9	21.2	21.2
Sel. 22a	3.1[1]	3.1[1]	4.2	26.0	6.3
Average	32.3	21.6	29.7	26.8	27.6

[1]The yield of the inoculated plants was greater than that of the uninoculated check plants.

Fig. 19—Relative resistance to bacterial wilt of Sel. 29a (foreground) and F. C. 19604 (background): Right, uninoculated; left, inoculated June 26 at height of 4 inches with a standard bacterial suspension. Photographed August 17, 1953.

tion, and this exception was related to variation in stand rather than to variation in wilt reaction.

In general the reduction in yield due to wilt was less in strains of *L. striata* (F.C.31057-5, F.C.31852, Kobe, Sel. 22a) than in strains of *L. stipulacea* (F.C.19604, Iowa 6, Korean, Rowan, Sel. 29a, Climax). This agrees with past results obtained in variety field trials under natural infection.

1954 Results: The 1953 test was repeated in 1954. The split plot arrangement included 10 strains of lespedeza (subplots) randomized within each of the five inoculation treatments (main plots). The experiment was replicated four times.

The test was seeded April 23. Good stands resulted and initial growth was satisfactory. Inoculations were made by the growing point method, May 26 for the juvenile stage and June 24 for the 4-inch stage of growth. The inoculum consisted of standard and ⅕-standard suspensions of the organism and 5 percent calcium hypochlorite solution for the check.

In late May when the juvenile leaf stage inoculations were made, soil moisture was still adequate for normal plant growth. Immediately following these inoculations deficient rainfall, combined with above normal daily temperatures, greatly retarded plant growth. By the time the 4-inch stage inoculations were made, the soil had become extremely dry and the daily

temperatures were high. These two factors almost stopped plant growth following the second group of inoculations. Two light irrigations were made and after each the plants became green but only limited growth took place. It was not until late in August that rain and cooler temperatures allowed the plants to resume normal growth.

When reduction in forage yield of the inoculation treatments was compared with that of the check treatment, it was found that yields were reduced only 11.6 percent (Table 19). This figure was lower than expected, since

TABLE 19 -- EFFECT OF WILT INOCULATION TREATMENTS ON THE FORAGE YIELD OF LESPEDEZA; FIELD STUDIES, 1954

Inoculation Treatment[1]	Average Yield of Ten Strains in Tons Per Acre[2]
Uninoculated (check)	1.31
Small-S	1.22
Small-1/5S	1.10
Large-S	1.15
Large-1/5S	1.15

[1]Small = early juvenile stage of growth; large = 4 inches tall; S = a standard suspension of bacteria.
[2]L.S.D. (.05) = 0.17 ton.

the yield reduction was 27.6 percent in the previous year. Differences in wilt resistance between strains were negligible because infection was slight; therefore, the data for individual strains are not presented. It appears from these results and from previous experience that susceptibility to bacterial wilt is influenced by the physiological condition of the plant. When plant growth was retarded by drouth, wilt damage was relatively small in all strains.

The multiple-pin method of inoculation was used in the field in 1954 (date not included) on three strains of lespedeza but the results were disappointing. Only a few leaflets developed wilt symptoms, and forage yields of the inoculated plants were as high as those of the check plants. Even F.C.-19604, which normally is very susceptible to the disease, escaped damage. It is probable that failure to obtain infection was due to heat and drouth interfering with the development of the disease.

DISCUSSION

Several aspects of the host-parasite relationship of bacterial wilt of lespedeza were investigated. It was felt that knowledge of the many factors involved would be helpful in developing suitable methods for breeding wilt resistant strains.

The mode of entry of the pathogen into the host was through wounds. The manner in which the plants were injured did not appear to be important provided some of the vascular elements were severed or ruptured and

exposed to the pathogen during or immediately following injury. Infection was obtained without difficulty by wounding either the roots or the foliage of young seedlings in the presence of the wilt bacterium. Infection could not be increased appreciably either by pouring a standard bacterial suspension over the foliage and around the roots of unwounded seedlings, or by germinating seeds with a water suspension of the organism. Spraying non-injured foliage of older plants with a bacterial suspension also failed to increase the disease appreciably.

Germination of lespedeza seed was not affected by the presence of the pathogen in the hulls. Only a small percentage of lespedeza seedlings contracted wilt during emergence, regardless of whether the seed was produced on healthy or on diseased plants. Seed which was soaked in a suspension of the causal organism appeared to germinate normally and remain free of infection as long as the emerging seedlings were not injured in any way. Limited data indicate that under natural conditions the seedlings first become infected through the cotyledons.

Data were obtained showing that the pathogen could over-winter in a virulent condition either in the seed hulls or in the stubble and debris of diseased plants. This provides a source of inoculum for infecting seedlings which become injured during germination or after emergence in early spring. Relatively few young seedlings become infected in volunteer stands. Perhaps the reason is that the amount of wounding generally is limited, and the concentration of bacteria generally is low. The initial infection usually takes place in or near the cotyledons, probably because it is here that natural injury most commonly occurs.

No evidence was found to support the theory that the pathogen may invade the embryo of the seed as it forms and later infect the seedling as it emerges. Prepared slides of seeds from diseased plants did not show bacteria present inside the seed, although they could be found readily in the seed hulls. The filtrate from such seed that had been hulled and surface sterilized did not produce the disease when used as inoculum. Attempts to isolate the wilt pathogen from the embryos of such seed were unsuccessful.

Wilting and death of leaves, branches, or entire plants is due in part to the destruction of xylem vessels by the pathogen. Histological preparations showed that many xylem vessels as well as other tissues were destroyed in advanced stages of the disease. It is possible, too, that bacterial plugging of xylem vessels and the direct action of bacterial toxins[1] may restrict water movement and contribute to wilting. Since wilted parts almost invariably were laden with bacteria, all three causes may contribute to the effect. Wilting occurred in a moist environment as readily as in a relatively dry one and once wilting appeared, the affected part could not be restored to a turgid condition.

[1]Gaumann, E. 1954. Endeavour 13:198-204.

Although the disease usually appeared in a few scattered plants during June, it did not become widespread until later in the season. When epiphytotics occurred, secondary spread seemed to be involved. Three possibilities of secondary spread were indicated, all of which involved wounding of the host: (1) grazing and trampling of animals, (2) feeding of grasshoppers and other insects, and (3) mowing, especially when the forage was wet.

Secondary spread and development of the disease appears to be greatly retarded by drouth. Extensive symptoms of the disease were not produced unless the plants were growing rapidly. Water deficiency in the plant reduces photosynthesis and cell turgidity and doubtless causes other changes, all of which may restrict the growth of the pathogen.

In general, lespedeza varied in reaction to bacterial wilt depending upon: (1) strain of lespedeza, (2) concentration or dosage of bacteria in the inoculum, (3) stage of plant growth at the time of inoculation, (4) method of wounding, and (5) environmental conditions for plant growth.

All strains of lespedeza usually were severely injured under conditions of extremely heavy infection. No strain was severely injured under conditions of mild infection. Conditions between the two extremes were most suitable for separating strains on the basis of resistance to the disease. Of the strains tested, F.C.19604 was the most susceptible, and selections 22a and 29a were the most resistant.

The severity of the disease increased with an increase in the concentration of bacteria in the inoculum within a certain range of concentration. With a given set of conditions there will be a minimum concentration, below which infection does not occur, and a maximum concentration above which there is no appreciable increase in severity of infection. The range in concentration that gives differences in infection could be expected to change if there were changes in the other factors which influence susceptibility.

The severity of the disease decreased when inoculation treatments were made at more advanced stages of growth. The cotyledonary stage was the most susceptible. At this stage, a $^{1}/_{100}$-standard dilution incited severe infection. The younger the plants the more difficult it was to detect differences in wilt resistance between strains.

The method of wounding plants under certain conditions influenced the severity of infection. In general, the more leaves or stems that were inoculated on a given plant, the more severe the infection. Cutting the cotyledons caused symptoms to develop earlier than did pinching, but the end result was about the same. Cutting or pinching two cotyledons resulted in more severe infection than cutting or pinching one cotyledon. There appeared to be little difference between severing the stems of plants immersed in a bacterial suspension or severing the stems immediately before immersing them. The growing point and multiple pin methods were the fastest

and easiest to perform of the foliage wounding methods tested. They produced differential disease reactions among lespedeza strains more nearly like those observed under natural infection than other foliage methods.

Root inoculation was less severe than foliage inoculation and under winter greenhouse conditions where a less severe method of inoculation was needed, the root method gave satisfactory results. Transplanting required in root inoculation is laborious. The sprayed-foliage clipping method was economical of labor but not of inoculum, and it did not give uniform infection between plants within strains. It probably would not be suitable for screening populations for resistant individuals.

It appears that susceptibility to bacterial wilt may be influenced by environmental factors which affect the physiological condition of the plant. Wilt damage usually was light in all strains when plant growth was retarded by drouth or low soil fertility. Wilt damage usually was severe in all strains when plant growth was modified by unfavorable light conditions. Satisfactory growing conditions are needed to bring out differences in susceptibility.

Lespedeza was more susceptible to bacterial wilt under winter light conditions than under spring or summer light conditions. Supplemental illumination should be provided during the winter and it should be sufficient to allow normal growth and development of the plants. Normal growth of lespedeza is difficult to obtain in the greenhouse in midwinter, for not only short day lengths and low light intensity, but also damping-off, variable soil moisture, and other environmental conditions frequently cause abnormal growth. Because of the limitations described, it may be necessary to conduct most of the work in the field in breeding for wilt resistant strains of lespedeza.

Measurement of the damage caused by wilt in stands of lespedeza presented a difficult problem. Considering the many factors involved, the determination of percentage reduction in forage yield appeared to be the most suitable method of measuring the damage caused by the disease.

Bacterial wilt reduced both the yield and the quality of lespedeza forage. Yield reductions ranged from about 5 percent for the more resistant strains to 50 percent for the more susceptible strains. Two factors associated with reduced yield were: (1) A marked stunting of infected plants, and (2) the dropping of infected leaves. Most of the loss in yield in the more resistant strains, such as Sel. 29a, was due to stunting. Susceptible strains, such as F.C.19604, not only were more severely stunted than were the more resistant strains, but also dropped a much larger percentage of their leaves. Since forage quality is closely related to leafiness, it is apparent that forage quality was lowered more in the susceptible strains than in the more resistant strains.

In order to estimate the damage caused by the disease in commercial

fields of lespedeza, a reliable index of yield reduction due to wilt is needed. A suggested approach to such an index follows: Artificially inoculate plants in an experimental plot; after the disease is well developed, harvest the plants and chop the foliage into small sections; after observing the stem sections for bacterial extrusions, determine the relationship between percent infected stems and reduction in forage yield due to the disease. Using this as a guide, it would be possible to sample commercial fields, determine percent of infected stems, and estimate the damage being incited by wilt.

To measure the wilt resistance of lespedeza strains in the greenhouse in winter, the following procedure is suggested: Use inoculum containing a standard suspension of bacteria (one test tube culture of the organism in 15 c.c. of sterile distilled water); use strong supplemental light to maintain a day length of at least 15 hours; inoculate plants 4 inches tall with the root tip immersion method (immerse the roots in a standard suspension of the pathogen and sever the root tips with scissors) or inoculate plants 4 inches tall with the growing point method (clip the growing point and petiole of the top trifoliate leaf with scissors dipped in $^{1}/_{25}$-standard suspension) or with the multiple pin method and a standard suspension. The greatest number of plants could be handled with the last two methods.

To measure the wilt resistance of lespedeza strains in the field in summer, use a standard suspension of bacteria and inoculate plants 4 inches tall with the growing point method. During periods of drouth, irrigation should be practiced to promote normal growth of plants and disease development.

The above procedures may be used for selecting resistant individuals in large populations. However, a more severe method probably should be used such as clipping both juvenile leaves as soon as they unfold, using scissors moistened in a standard suspension of bacteria. Root inoculation in the cotyledonary stage should give similar results but would require more labor and space.

The most severe method of inoculation that might be used consists of wounding the freshly opened cotyledons with scissors or forceps dipped in a standard suspension. Under conditions of water, light, and temperature that promote rapid, normal growth of plants, resistant individuals may survive, especially when only one cotyledon on each seedling is wounded. Under conditions less ideal for growth all may be killed, including individuals that would be resistant with less severe methods.

To eliminate escapes, regardless of the method used to initiate infection, it is wise to continue inoculating the new leaves as they unfold on surviving plants.

Inheritance studies of wilt resistance would be difficult using lespedeza strains presently available. Resistance in these strains is influenced greatly by external factors, making it difficult to separate individuals into sharply

defined classes. By progeny testing individuals in segregating generations, it should be possible to classify them for wilt resistance with reasonable accuracy. Inoculation should be by the growing point method as suggested above for field studies.

A large collection of lespedeza strains was grown in the field over a period of several years. This afforded an opportunity to observe the occurrence of wilt in a broad sample of germ plasm. The most susceptible strains were among the earliest maturing. The most resistant strains were among the latest maturing. Inheritance studies should be designed to study linkage of maturity with resistance to wilt. It is important to know whether or not wilt resistance can be combined with early maturity to give the resistant early strains so greatly needed in Missouri. If the best resistance now available turns out to be unsatisfactory in degree of protection or in ease of transfer, then the search should continue for a better type of resistance.

SUMMARY

1. Bacterial wilt, *Xanthomonas lespedezae* (Ayers *et al.*) Starr, is one of the most serious diseases of lespedeza in Missouri, and has limited greatly the performance of the crop under certain conditions.

2. Studies were made of the host-parasite relationship and of inoculation and evaluation methods as they relate to breeding for wilt resistance in annual lespedeza.

3. The pathogen entered the host through wounds and spread through the vascular system. Many xylem vessels as well as other tissues were destroyed in advanced stages of the disease. The pathogen traveled more rapidly in the xylem vessels of susceptible strains than in those of resistant strains.

4. Hulls of stored seed, stems, and debris of diseased plants left in the field during the winter contained virulent bacteria as late as April of the following year. These are important means of overwintering. Attempts to demonstrate that the pathogen was carried inside the seed were unsuccessful.

5. The amount of primary seedling infection which occurs in susceptible strains from natural causes may amount to less than 3 percent. It appears that primary infection usually occurs in or near the cotyledons of very young seedlings. It is possible for it to occur in any part of the plant.

6. When natural epiphytotics occurred, secondary spread appeared to be involved. Three possibilities of secondary spread were indicated, all of which involved wounding of the host: (1) The grazing and trampling of animals, (2) the feeding of grasshoppers and other insects, and (3) mowing, especially when the forage was wet.

7. A measure of the wilt resistance of lespedeza strains was obtained by determining the yield reduction due to wilt on a percentage basis. Yield

reduction following field inoculation ranged from about 5 percent for the more resistant strains to 50 percent for the more susceptible strains. Two factors associated with reduced yield and quality of forage were stunting of growth and dropping of infected leaves.

8. A wilt index is suggested as a guide for estimating the yield reduction due to wilt in commercial fields.

9. A controlled set of conditions was essential for the production of uniform epiphytotics of wilt. Several factors influenced the severity of infection: (1) Concentration of bacteria in the inoculum, (2) stage of plant growth, (3) method of wounding, and (4) environmental conditions for plant growth, particularly light and soil moisture. The differential reaction of plants to inoculation with wilt is influenced by these factors through their effect on the severity of infection. Extremely severe or extremely mild infection did not differentiate satisfactorily between strains or individual plants.

10. Various procedures are suggested in the Discussion for measuring resistance to wilt in lespedeza strains under greenhouse and field conditions and for selecting resistant individuals in large populations. These procedures are designed to produce a given severity of infection, taking into account the influence of several factors upon the severity of the disease.

11. Possibilities exist for further improvement in procedure for measuring resistance to wilt among strains and among individual plants. The goal is effective procedures that permit rapid inoculation of large numbers of plants with maximum economy of labor and inoculum.

12. Studies of the inheritance of wilt resistance and the linkage relationship of wilt resistance and plant maturity are needed.

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